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Two active and differently *N*-glycosylated isoforms of human ST3Gal-V are produced from the placental mRNA variant by a leaky scanning mechanism

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ABSTRACT

Previously, we identified a human ST3Gal-V mRNA variant peculiarly characterized by the presence of a translational start codon localized up-stream and in-frame with the one that is usually considered as unique translation initiation site in the human gene. In this study we demonstrate, by cDNA transfection experiments, mutational analyses, enzyme activity assays, and endoglycosidase-H treatments, that the in vivo expression of this transcript gives rise to two human ST3Gal-V isoforms with distinct characteristics. Produced by a leaky scanning mechanism, they carry different *N*-glycan structures and exhibit differences in their GM_3 synthase activity that might be relevant for the modulation of GM_3 cellular content.

1. Introduction

Gangliosides, a large and structurally heterogeneous family of sialic acid (NeuAc)-containing glycosphingolipids, are ubiquitous components of all eukaryotic cells. Mainly located in the plasma membrane, gangliosides are not only structural components of plasma membranes, but they also participate to cell surface events, including cell signaling transduction, subcellular targeting, and cell adhesion [1]. In addition, remarkable changes of ganglioside distribution in cells and tissues have been reported during various both physiological and pathological cell processes, such as cell proliferation and differentiation, apoptosis, development and oncogenesis [1–4].

The ganglioside biosynthesis is a finely regulated process [5], and the enzyme which plays a key regulatory role is the GM_3 synthase, also named ST3Gal-V or Sial-T1 (CMP-NeuAc: lactosylceramide α2,3-sialyltransferase, EC 2.4.99.9). It catalyses the transfer of a NeuAc residue to the terminal galactose of lactosylceramide with synthesis of the ganglioside GM_3, the common precursor for nearly all of the naturally occurring ganglioside species [5]. GM_3 is also involved in type 2 diabetes [6], and an homozygous loss-of-function non-sense mutation in the human ST3Gal-V gene is cause of an autosomal recessive infantile–onset symptomatic epilepsy syndrome [7].

ST3Gal-V is described as a Golgi membrane-bound glycoprotein, and the overall picture of the subcellular location of the different steps of ganglioside biosynthesis shows the presence of GM_3 synthase activity in both the cis/medial Golgi and the trans Golgi network [8,9]. The different location of ST3Gal-V could contribute to subtly control the cellular ganglioside profiles: whereas the ST3Gal-V localized in the proximal Golgi seems to be preferentially involved in the synthesis of simple gangliosides (i.e., GM_3 and GD_3), the enzyme with GM_3 synthase activity localized in the distal Golgi seems to be important in the synthesis of more complex GM_3-derived gangliosides (i.e., GD_{1a}, GT_{1a}, GT_{1b}, GQ_{1b}) [8–11].

Several efforts have been done to investigate the human ST3Gal-V gene expression in different tissues, and to examine the mechanism for transcriptional activation of ST3Gal-V gene [12–16]. Altogether these studies indicated that the expression of the human ST3Gal-V gene is mainly regulated at transcriptional level resulting in the production of four mRNA variants that differ in their 5′-untranslated region, but contain a coding region producing a protein identical in both length and primary sequence [16–18].

In 2006 we reported the identification of a novel variant of human ST3Gal-V transcript providing the first evidence of the possible existence of two isoforms of human GM_3 synthase [19]. Isolated from human placenta and then identified in HL60-undifferentiated cells, this mRNA isoform is peculiarly characterized by the presence of a translational start codon localized up-stream and in-frame with that one usually considered as unique translation initiation site in the human gene [19].

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In the present study, we demonstrate that the in vivo expression of this mRNA variant gives rise to two differentially extended human ST3Gal-V isoforms. They are produced by leaky scanning mechanism and exhibit differences in their GM_3 synthase activity. Moreover, the two human ST3Gal-V isoforms carry *N*-glycan structures with different sensitivity to endonuclease-H treatments that might be relevant to define GM_3 levels in the cell.

2. Materials and methods

2.1. Expression plasmid construction

The plasmids pP-Rc/CMV and pT-Rc/CMV, containing two differently extended open reading frame (ORF) of the human placental ST3Gal-V mRNA variant (GenBank, accession no AY152815), were prepared as indicated [19]. pP-Rc/CMV is the construct containing the "short" ORF (named P insert), that is the cDNA region extending from nucleotides 97 to 1287 of the sequence in GenBank containing only the downstream translation start codon (i.e., AUG^{153}). pT-Rc/CMV is the construct containing the "long" ORF (named T insert), that is the cDNA region extending from nucleotides 1 to 1287 of the sequence in GenBank containing both the in-frame translation start codons (i.e., AUG^{54} and AUG^{153}). Nucleotides and AUG codons are numbered according to Ref. [19].

To express the carboxy-terminally c-myc epitope-tagged human ST3Gal-V, the "short" ORF and the "long" ORF of the human ST3Gal-V cDNA were isolated from the plasmids pP-Rc/CMV and pT-Rc/CMV, respectively, by double digestion with HindIII and BglII, blunted with Klenow, and ligated into the blunted, calf intestinal phosphatase-treated BglII site of the vector pcDNA™ 3.1/myc-His(−)A (InVitrogen, Carlsbad, CA, USA). The identity of each construct (named pP-myc and pT-myc, respectively) was confirmed by restriction mapping and the structure of the insert junction was ascertained by DNA sequencing (M-Medical Sequencing Service, Firenze, Italy).

2.2. Mutant construct preparation

Mutated constructs, called pMut54-myc and pMut153-myc, were prepared by specific PCR amplifications using pT-myc construct as template and two couples of specific mutated oligonucleotides as primers. To obtain pMut54-myc we used the following: 5′-GCCACCCAGGAC**G**TGGCTTCTGTTCCAATGCCAAGT G-3′ and its reverse complementary 5′-CACTTGGCATTGGAACAGAAGCCA**C**GT CCTGGGTGGC-3′; to obtain pMut153-myc the following: 5′-CCGA GCTCAAAGCAAG**G**TGAGAAGGCCCAGCTTG-3′ and its reverse complementary 5′-CAAGCTGGGCCTTCTCA**C**CTTGCTTTGAGCTCGG-3′. Mutated nucleotides are in bold and underlined. The PCR reactions (50 μl), containing 20 mM Tris–HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA, 0.2 mM dNTP, 1.5 units of Pfu DNA polymerase and 125 ng of each specific mutated primer, were carried out according the following conditions: for mutant pMut153-myc, 25 cycles, at 95 °C-30 s, 62 °C-1 min, 72 °C-14 m; for mutant pMut54-myc, 25 cycles, at 95 °C-30 s, 72 °C-14 min. The presence of the correct mutation was ascertained by DNA sequencing in both directions.

2.3. Cell culture, transfection and tunicamycin treatment

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM *l*-glutamine, 1% Na–pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C. Transfections were carried out on 4×10^5 cells using 4 μg of indicated plasmid DNA and 21 μl of Arrest-In™ Transfection Reagent (Open Biosystems, Huntsville, AL, USA) according to the manufacturer's instruction. After 48 h, the cells were harvested and lysed.

In tunicamycin experiments, COS-7 cells were transfected with indicated plasmid DNA as previously described. Six hours after transfection, the medium was replaced with fresh culture medium supplemented or not with 1 μg/ml tunicamycin (Sigma). Twenty-four hours later the cells were harvested and used to measure the enzymatic activity.

2.4. Cell lysates and glycosidase treatments

Mutant-transfected COS-7 cells were lysed in culture dishes with RIPA buffer for 20 min at 4 °C on a rotary shaker. Collected crude lysates were allowed to stand for another hour on ice and then centrifuged 10 m at 10 000×*g* at 4 °C to remove nuclei and large cell debris. Proteins were estimated according to the Lowry's method [20].

For peptide *N*-glycosidase F (PNGase-F) digestions, total cell lysates (∼500 μg of proteins) in 50 mM sodium-phosphate, pH 7.5 and 1% SDS were incubated for 10 m at 100 °C. After addition of 5 U PNGase-F (Roche, Basel, Switzerland), the reaction was carried out at 37 °C for 18 h. For digestions with endoglycosidase-H (Endo-H), total cell lysates (∼500 μg of proteins) in 50 mM sodium-citrate, pH 6 and 1% SDS were incubated for 30 m at 65 °C. After addition of 16 mU Endo-H (Roche, Basel, Switzerland), the reaction was carried out for 1 h at 37 °C.

2.5. Immunoblotting analysis

Total cell lysates (∼300 μg of proteins) from control and transfected COS-7 cells, microsomal-enriched protein fractions (∼100 μg of proteins) from pMut54 and pMut153 transfected-cells before and after tunicamycin treatment, and *N*-glycosidase treated samples were resolved by 12% SDS–PAGE, blotted on PVDF membrane (Bio-Rad, Richmond, CA, USA), and immunodetected with anti-myc monoclonal antibodies (clone 9E10, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:500. Anti-myc immunoreactive proteins were then pointed out with HRP-conjugated anti-mouse IgG polyclonal antibodies at a dilution of 1:2000 dilution and ECL Western blotting detection reagents (GE Healthcare, Little Chalfont, UK).

2.6. Enzymatic activity assay

The enzymatic activity was determined by an in vitro radioactive assay as previously described [21], using the microsomal-enriched protein fraction as enzyme source. Briefly, control and mutant transfected COS-7 cells, before and after tunicamycin treatment, were lysed in 0.32 M sucrose. After a low speed centrifugation to remove nuclei, the supernatant was centrifuged at 100 000×*g* for 1h at 4 °C. To measure enzyme activity, the reaction mixture (50 μl), containing 0.1 M sodium cacodylate pH 6.4, 10 mM $MgCl_2$, 0.2% Triton CF54, 1 mM CMP-[^{14}C]-sialic acid (0.2 μCi, Amersham), 1 mM acceptor glycolipid substrate LacCer (Alexis Biochemicals, San Diego, CA, USA), and membrane preparation (∼100 μg of proteins), was incubated at 37 °C for 2 h. The amount of incorporated [^{14}C]-NeuAc were determined after paper descending chromatography by liquid scintillation counting.

3. Results and discussion

All the human ST3Gal-V mRNA variants identified in the adult and fetal brain [16,18] and in TPA-treated HL60 cells [17] contain an identical ORF encoding a protein of 362 amino acids exhibiting GM_3 synthase activity. However, the identification, in the human

placenta and undifferentiated-HL60 cells, of a mRNA variant containing an additional translation start codon (from now on called $A^{54}UG$), located up-stream and in-frame with the translation initiation site in all the other human ST3Gal-V mRNA variants (from now on called $A^{153}UG$), strongly suggested the existence of a different isoform of the enzyme [19]. This new isoform, consisting of 395 amino acids ("long" form), would retained unaltered the large COOH-terminal domain of 329 amino acids containing the catalytic site, the single transmembrane domain of 18 amino acids and the two potential *N*-glycosylation sites of the 362 amino acid protein (or "short" form), but it would have an additional NH_2-terminal extension of 33 amino acids.

To begin the characterization of the protein encoded by the human placental ST3Gal-V mRNA, the plasmid pT-myc, which directs the expression of the protein tagged with the antigenic c-myc epitope at its C-terminus, was transfected into COS-7 cells. In agreement with the molecular mass of the protein expressed in vitro [19], western blotting analysis of cell lysates from pT-myc transfected-cells with anti c-myc antibodies clearly shows the presence of an immunoreactive protein migrating in SDS–PAGE with the molecular mass of ~48 kDa (Fig. 1, lane T); this corresponds to the molecular mass predicted from the amino acid sequence from the $A^{54}UG$ codon of the cloned cDNA (~44.5 kDa) plus two possible N-linked oligosaccharides [19]. However, in lane T it is also evident a ~45 kDa immunoreactive polypeptide that, interestingly, exhibits an electrophoretic mobility similar to that of the protein expressed in cells transfected with the plasmid pP-myc (Fig. 1, lane P), the construct which directs the expression of the "short" human ST3Gal-V tagged with the antigenic c-myc epitope at its C-terminus. No proteins of ~48 and/or ~45 kDa are in extracts from mock-transfected COS-7 cells (Fig. 1, lane C).

The ~45 kDa protein detected in pT-myc expressing cells could be either the unglycosylated form or the proteolytic product of the ~48 kDa protein, since the difference of ~3 kDa between the two polypeptide chains is consistent with the presence of two possible N-linked oligosaccharide chains as well as with the 33 amino acids which constitute the NH_2-terminal extension of the "long" human ST3Gal-V isoform. On the other hand, another equally good possibility is that the protein with the lower molecular mass derives from the usage of the codon $A^{153}UG$, instead of the codon $A^{54}UG$, as translational start site.

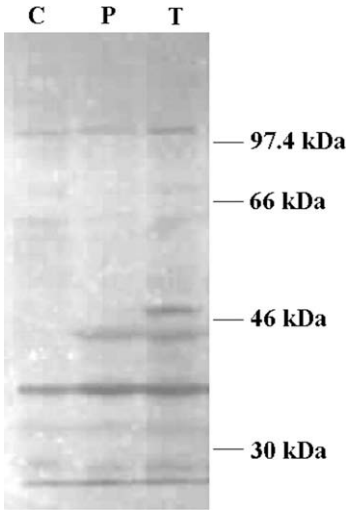

Fig. 1. Western blot analysis of the human placental ST3Gal-V mRNA expression. Total cell extracts from COS-7 cells transfected with the expression vector alone (lane C) or with pP-myc (lane P) or pT-myc (lane T) constructs were fractionated by SDS–PAGE and immunodetected with anti c-myc antibodies.

To examine this issue an approach based on mutational analyses was carried out and two mutated constructs, pMut54-myc and pMut153-myc, were generated. pMut54-myc is the construct where the A to G transition at position 54 of the cDNA results in the elimination of the translational start codon $A^{54}UG$ leaving the $A^{153}UG$ unaltered. pMut153-myc is the construct where the A to G transition at position 153 of the cDNA introduces a Met to Val substitution in the protein sequence eliminating the $A^{153}UG$ codon, but leaves unaltered the $A^{54}UG$ codon. As clearly evidenced by immunoblotting analysis, pMut54-myc expressing cells show the

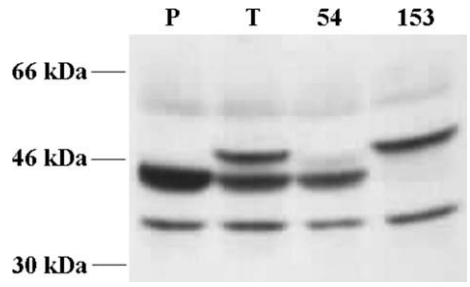

Fig. 2. Western blot analysis of the selective expression of the two human ST3Gal-V isoforms. Total cell extracts from COS-7 cells transfected with the normal pP-myc or pT-myc constructs (lanes P and T, respectively) or with the "mutated" pMut54-myc or pMut153-myc constructs (lanes 54 and 153, respectively) were fractionated by SDS–PAGE and immunodetected with anti c-myc antibodies.

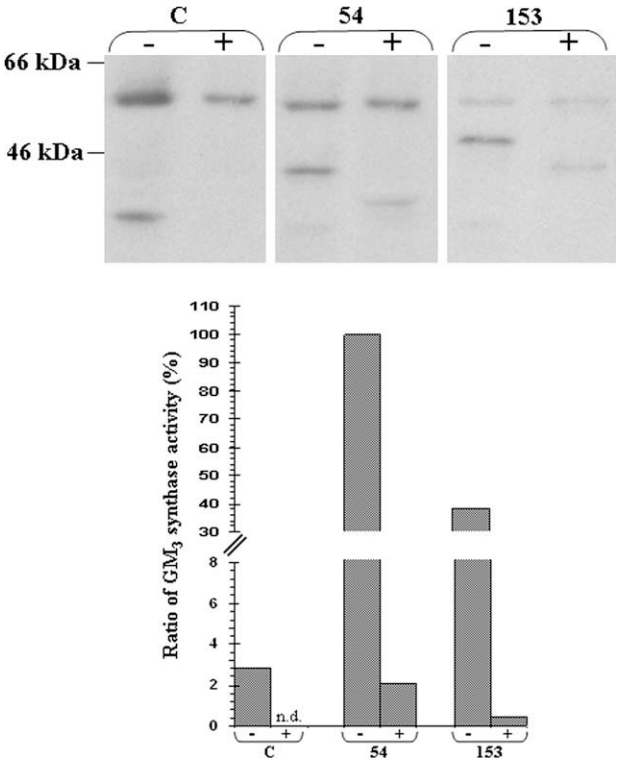

Fig. 3. GM_3 synthase activity of human ST3Gal-V isoforms and effects of tunicamycin treatment. Microsomal-enriched protein fractions from control COS-7 cells (samples C) and from pMut54-myc or pMut153-myc expressing cells (samples 54 and 153, respectively), before (−) and after (+) tunicamycin treatment, were analysed by immunoblotting with anti-myc antibodies (upper gel), and then used to measure the enzyme activity (histogram). Data of the enzyme activity in pMut54-myc and pMut153-myc expressing cells are expressed as relative to the corresponding protein levels determined by immunoblotting with anti-myc antibodies. Data of enzyme activity levels in control COS-7 cells are expressed as relative to the enzyme activity in pMut54 expressing cells. n.d. = not detected.

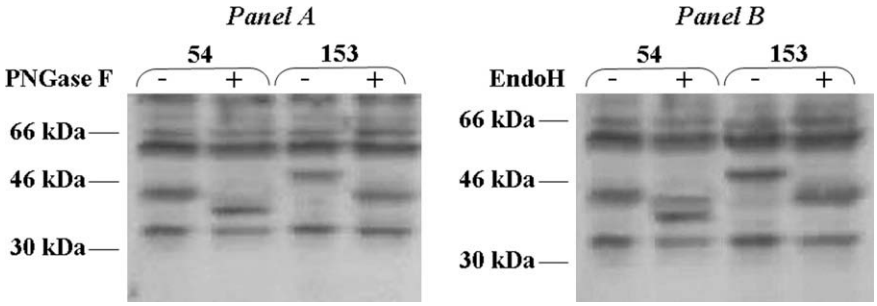

Fig. 4. PNGase-F and Endo-H sensitivity of human ST3Gal-V isoforms. *N*-glycosidase sensitivity was analyzed in total cell extracts from pMut54-myc or pMut153-myc COS-7 cells expressing cells (lanes 54 and 153, respectively) treated with (+) or without (−) PNGase F (Panel A) or Endo-H (Panel B), by SDS–PAGE separation of digestion products, followed by immunoblot with anti-myc antibodies.

presence of only one immunoreactive protein with molecular mass consistent with the expected molecular mass of the protein whose translation start codon is the $A^{153}UG$ (i.e., ~45 kDa) and migrating as the smaller polypeptide in pT-Myc transfected-cells (Fig. 2, compare lane 54 to lane T). Likewise, pMut153-myc expressing cells (Fig. 2, lane 153) show the presence of only one immunoreactive polypeptide: it migrates in SDS–PAGE as the longer protein in pT-myc expressing cells (Fig. 2, lane T) and its molecular mass of ~48 kDa is consistent with the expected molecular mass of the protein whose translation start codon is the $A^{54}UG$ codon.

Therefore, these results demonstrate that the polypeptides of ~48 and ~45 kDa detected in pT-myc transfected-cells are produced by the alternative usage of the $A^{54}UG$ and $A^{153}UG$ codons, respectively, and demonstrate that two isoforms of human ST3Gal-V can be co-expressed from a unique transcript. Up to now we do not know how occurs the AUG codon selection in pT-myc-expressing cells, and any hypothesis would be too speculative at this point of knowledge. It is certain that the two isoforms in pT-myc-expressing cells are not always expressed in the same relative ratio. In fact, by comparing Figs. 1 and 2 we can appreciate that the lower band in lane T of Fig. 2 is clearly stronger than the upper one, but this does not occur in lane T of Fig. 1 where the intensity of the bands is very similar. The variability of the relative amount of the two isoforms in transiently transfected-cells suggests a possible "casual" selection of the translational start codon. However, further experiments are needed to exclude the existence of one or more cell-specific mechanisms regulating the process.pMut54-myc and pMut153-myc transfected-cells were also examined to assess the GM_3 synthase activity of each isoform. Results are in Fig. 3: pMut54-myc transfected-cells exhibit higher levels of GM_3 synthase activity than pMut153-myc transfected-cells (~2.7 fold). Of note, the amount of the respective c-myc-tagged expressed proteins in the microsomal-enriched fractions used as enzymatic sources were at similar levels [compare lanes 54 (−) to 153 (−) in the immunoblotting of Fig. 3].

As the influence of *N*-glycan structures on enzyme activity has been demonstrated for several glycosyltransferases [22], to examine whether the GM_3 synthase activity of both isoforms was dependent on their *N*-glycosylation, pMut54-myc and pMut153-myc overexpressing cells were treated with tunicamycin, an inhibitor of the formation of dolichol pyrophosphate-N-acetyl glucosamine resulting in the block of the *N*-glycosylation process, and microsomal-enriched protein fractions were used to measure the enzyme activity. As expected, the tunicamycin treatment abolishes the *N*-glycosylation of both isoforms which are detected as a single band with a molecular mass lower than that of the corresponding protein from untreated cells [compare lanes 54 (+) and 153 (+) to lanes 54 (−) and 153 (−), respectively, in the immunoblotting of Fig. 3], and, more importantly, it causes a remarkable reduction in their enzyme activity (Fig. 3). Interestingly, a significant decrement of the over-expressed protein levels seems to occur after tunicamycin treatment, suggesting a possible involvement of *N*-glycans in the stability of these proteins.

To characterize the structures of *N*-glycan chains bound to both isoforms cell lysates from mutated transfected-cells have been analyzed by treatments with PNGase-F and Endo-H. Digestion products were then analysed by separation on SDS–PAGE, followed by immunodetection with anti-c-myc antibodies.

Immunoblotting after PNGase-F treatment (Fig. 4, Panel A) clearly show that the ~45 kDa protein in pMut54-myc expressing cells (lane 54+) and the ~48 kDa protein in pMut153-myc expressing cells (lane 153+) are entirely susceptible to PNGase-F digestion resulting in a reduction in size by ~3 kDa. In contrast, immunoblotting analysis of cell lysates after Endo-H treatment (Fig. 4, Panel B) shows that, whereas the ~48 kDa protein in pMut153-myc expressing cells is entirely sensitive to Endo-H (compare lane 153− to lane 153+), the ~45 kDa protein in pMut54-myc expressing cells is only partially susceptible to Endo-H digestion (compare lane 54− to lane 54+), indicating that both human ST3Gal-V isoforms are *N*-glycosylated and that, the two isoforms of human ST3Gal-V carry different *N*-glycan structures.

In conclusion, this report provides the first evidence of the existence, also in human cells, of a system which produces two ST3Gal-V isoforms having distinct characteristics from an unique transcript, and it prompts further studies addressed to establish how the expression of the isoforms is regulated, and if and how the expression of one or both isoforms might be of critical importance for the regulation of GM_3 biosynthesis under pathological and physiological conditions, as recently hypothesised for the mouse ST3Gal-V [23].

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